ISOLATION AND CHARACTERIZATION OF A SYNCYTIUM-FORMING VIRUS OF RACCOONS

By

STANLEY NICOLAS LAHAM

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To my mother, for her love, devotion and unshakeable faith in my capabilities

and

To my father, for his patient understanding and constant support.

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Abstract of Dissertation Presented to the Graduate Council of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

ISOLATION AND CHARACTERIZATION OF A SYNCYTIUM-FORMING VIRUS OF RACCOONS

By

Stanley Nicolas Laham

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A virus that shares all of the distinctive characteristics of the syncytium-forming viruses of simian, bovine, feline, hamster and human origin was isolated from the tissues of raccoons. It was found to have a wide *in vitro* host range with giant cell formation as its most characteristic cytopathic effect. Viral antigen was detectable in the nuclei of these cells by indirect immunofluorescence.

Persistent infection was experimentally induced in rabbits and an antigen extracted from infected cells was used to detect precipitating antibodies in infected animals. A serological survey of Florida raccoons revealed a wide geographical distribution of the virus.

An immunofluorescent assay was developed to quantitate the agent. The virus was inactivated by the lipid solvents ether and chloroform indicating it to be enveloped. It was

shown to incorporate ^{3}H uridine but not ^{3}H-thymidine and this incorporation was inhibited by low levels of actinomycin D. Furthermore, its replication was blocked by bromodeoxyuridine, indicating a DNA replicative stage which was confirmed by demonstration of virion associated reverse transcriptase activity. The virus banded at a density of 1.18 g/cm^3 in sucrose gradients.

Electron microscopic studies revealed an enveloped spherical virus with a diameter of 85 to 105 nm. The viral core (58 to 64 nm in diameter) was composed of an electron opaque outer ring with an electron-lucent center. The envelope was covered with small projections, and the virus was seen to mature by budding of preformed nucleoids through the plasma or intracytoplasmic membrane.

I. INTRODUCTION

When an organism or group of organisms is to be given a functional appellation, a specific designation should be used which conveys its uniqueness and separates it from other related entities. If the name chosen does not accomplish this purpose, then further clarification becomes necessary. That is the problem with the term "syncytium-forming virus."

There are many other viruses that cause syncytium formation in vitro including members of the orthomyxo-, paramyxo-, corona-, oncorna-, and herpes virus group. The syncytium-forming viruses (also referred to as foamy agents) are RNA viruses having virion associated RNA-dependent DNA polymerase. They are present in man and a variety of animals, causing persistent infection with no known associated disease (17, 25). The human and bovine respiratory syncytial virus are not included in this group. Although they share certain characteristics with the foamy viruses (e.g., cell association, lack of hemagglutinin and syncytium-formation in cell cultures), they do not

produce an antigen detectable in the nuclei of infected cells, a principal feature of the syncytium-forming viruses.

In this text, the terms "syncytium-forming virus" and "foamy virus" will be used interchangeably. Recently, these agents have been recognized as a unique group and classified as the subfamily Spumavirinae of the RNA virus family Retroviridae.

II. BACKGROUND REVIEW

The first foamy viruses to be studied were of simian origin. Enders and Peebles (7) in 1954 reported the spontaneous foamy degeneration of monkey kidney cell cultures. However, apart from describing the cytopathic effect in those cultures, no other data was given concerning their agent.

A year later (during attempts to adapt dengue virus to monkey kidney cell cultures), Rustigian *et al.* (28) isolated an agent from kidneys of rhesus monkeys (*Macaca mulatta*) and designated it Simian Foamy Virus (SFV) Type I. They described the degenerative process in the kidney cell cultures as follows: "There were areas in which individual cells were no longer visible but appeared to be made up of degenerated and perhaps fused cells. . .clusters of nuclei could be distinguished. The multiple nuclei, . . . suggested large fused syncytial structure. There appeared, at the same time, variable sized vacuoles which in some instances were more prominent than the syncytial structures" (p. 10). This dilatation of vacuoles produced the foamy appearance in their cell cultures.

They inoculated other cell types with filtered supernatant fluids from their degenerated cultures. Similar cytopathic effects were obtained in all instances except in a mouse kidney cell culture which was unaffected by the agent. With HeLa epithelial cells, they made the following observations: "Of much greater contrast to reaction in monkey cultures was the absence of vacuolation. Degeneration seemed to consist mainly of a gradual increase in size and number of lytic-like areas. . . .At the same time multinucleated cellular masses representing either giant cells or syncytial structures became more prominent" (p. 10). Thus, syncytium-formation seemed to be a more universal manifestation of the virus than did vacuolation of infected cells.

Simian Foamy Virus Type II was isolated in 1961 from cynomolgus monkeys (Macaca fascicularis) (20). Since that time 7 other serotypes of SFV's have been recovered from various species of monkeys and apes (21, 17, 18, 20, 31) giving a total of 9 serotypes recognized to date. Cross-neutralization studies have shown each serotype to be antigenically distinct (8, 13, 18).

It was not until 1969 that a syncytium-forming virus was isolated from a non-primate species. Malmquist et al. (24) designated as bovine syncytial virus a foamy agent

which they recovered from both normal and lymphosarcomatous cattle. It induced syncytia formation in bovine embryonic spleen cells (BESP) and the rabbit cornea cell line SIRC. Virus was isolated from direct cell cultures initiated from lymph nodes and spleens of lymphosarcomatous cattle. It was also recovered by co-cultivation of the cellular component of milk and buffy coat cells of both normal and lymphosarcomatous animals with BESP or SIRC cells.

The investigators developed an immunodiffusion test to detect precipitating antibodies in infected cattle. The antigen used in this test was prepared by sonically disrupting heavy suspensions of cells from highly infected cultures. They pointed out that "the presence of antibodies demonstrable by the immunodiffusion test did not terminate the carrier state" (p. 192) in infected cattle.

The bovine virus seemed to be very cell associated and difficult to transmit with cell-free fluid. Nuclei of infected cells exhibited viral antigens detectable by immunofluorescence. Electron microscopy revealed a viral core 35 to 45 nm in diameter enveloped by an outer membrane covered with surface projections. The complete virion had an overall diameter of 90-115 nm.

A feline syncytium-forming virus was isolated from normal cats and cats with a variety of illnesses such as feline infectious peritonitis, neoplasms and urolithiasis (12, 13, 16, 19, 23, 27). Kasza et al. (23) isolated a syncytium-forming agent from a sarcoma of a cat in an established canine melanoma cell line. Importantly, they observed that no cytopathology was evident in these cell cultures in the first passage. After three passages by sub-cultivation, 50 to 80% of their cells became involved in syncytium-formation. The authors pointed out that the cytopathic effect appeared most rapidly when cell monolayers were infected before they were completely confluent.

Achong et al. (1) recovered a human foamy agent from a nasopharyngeal carcinoma. The virus was shown to share all the major characteristics of the syncytium-forming viruses of the other species (8). It was not neutralized by antisera to any of the known animal foamy agents demonstrating that it is immunologically distinct. Its cytopathogenicity was inhibited by addition of BUDR to culture fluids suggesting a DNA replicative stage. By electron microscopy, the virus could be seen to mature by entry of a preformed nucleoid into a bud at the cell membrane. The completed virions

were covered with radiating spikes. The authors remark that the morphology and morphogenesis of their virus was very similar to that of the simian, bovine, and feline agents. Since these viruses were unassignable to any established morphological family, Epstein et al. (8) suggested that they could represent members of a new group.

Parks and Todaro (25) have pointed that the syncytium-forming viruses of feline, bovine and simian origin are members of a unique group. Although all are able to cause dilation of vacuoles producing a foamy appearance in the cytoplasm of infected cells the authors state that "the syncytium-forming ability of these viruses appear to be a more characteristic manifestation of their cytopathology" (p. 676). Table I lists the known established syncytium-forming viruses. (A rabbit syncytial virus has been described but not definitively characterized.) A common group antigen has not been demonstrated.

Table I

Foamy Viruses (17)

Simian foamy viruses (9 serotypes)
Bovine syncytium-forming virus
Feline syncytium-forming virus
Hamster foamy virus (9)
Human foamy virus

The syncytium-forming viruses have a buoyant density of 1.16 to 1.18 g/cm^3 and have been shown to be sensitive to chloroform and ether (8, 16, 17, 25). Low concentrations of bromodeoxyuridine (BUDR) in culture fluids markedly decrease virus yield (8, 25). Parks et al. (26) have shown incorporation of tritiated uridine but not thymidine with simian serotype 3. Furthermore, uridine incorporation is inhibited by actinomycin D (26). This is a characteristic of the retroviruses which go through a DNA replicative intermediate stage via reverse transcriptase. The simian, bovine and feline agents have been shown to have reverse transcriptase activity (17, 26, 29), resulting in their classification as a subfamily of the Retroviridae (3).

A plaque-forming assay was developed for simian foamy viruses Types I and III but the same method proved unsuccessful with the bovine agent (6). Generally syncytium-forming viruses replicate to very low titers and require several passages of infected cells before the cytopathic effect becomes evident (17, 25). Park and Todaro (25) aptly remark that biological studies are hindered by their low level of infectivity, marked lability and lack of a quantitative *in vitro* assay procedure.

The foamy viruses seem to be ubiquitous in nature and cause persistent infection in their hosts. For example, Hackett *et al.* (16) reported isolation of feline syncytium-forming virus (FeSFV) from 90% of cats in their laboratories during the first four months of 1969. Lymphocytes have been shown to be infected in the case of bovine agent (14, 24). A characteristic host response to infection by these agents is formation of virus-specific precipitating antibodies. Gaskin and Gillespie (12) working with FeSFV have shown that the presence of precipitating antibodies is indicative of chronic infection. Malmquist *et al.* (24) have demonstrated the same with the bovine agent.

The syncytium-forming viruses infect a wide range of cells *in vitro*. They propagate in epithelial and

fibroblastic cells of numerous mammals tested. Parks and Todaro (25) and Hooks and Gibbs (17) list the characteristics of the syncytium-forming (foamy) viruses as follows:

1. Wide *in vitro* host range for cell species and cell types.
2. Syncytium formation as characteristic *in vitro* cytopathic effect.
3. Inhibition of replication by BUDR and dactinomycin.
4. Necessity for actively replicating cells in order to initiate infection.
5. Multiplication to low levels of infectivity (10^2 to 10^5 $TCID_{50}$/ml).
6. Virion--associated reverse transcriptase.
7. High resistance to inactivation by ultraviolet light treatment.
8. Production of viral antigen in the nuclei of infected cells detectable by immunofluorescence.
9. By electron microscopy, have nucleoids 30-35 nm in diameter which are usually seen in the perinuclear cytoplasm. Are 100 to 140 nm in diameter after budding through the plasma membrane with no clearly observable symmetry. The enveloped virions are covered with surface projections or spikes.
10. No cellular transformation *in vitro* or tumor production *in vivo* yet observed.

Work with the foamy viruses of various species has shown that they are common in nature, cause persistent infections and persist in the leukocytic cells of their host.

Scott (30) concluded that FeSFV was a nuisance to virologists, oncologists, and vaccine producers working with feline cells. Hackett and Manning (15) point out that since this agent is strongly cell-associated and replicates to such low titers it can easily be overlooked. It is now feared that some of the early poliovirus vaccines were contaminated with simian foamy viruses since neutralizing antibodies to SFV type III were detected in one out of 20 sera of human vaccinees checked (22). There should be a concerted effort to uncover and characterize these viruses so that virologists, cancer researchers, and vaccine producers can be made aware of them.

This dissertation is concerned with the characterization of a previously undescribed syncytium-forming virus of raccoons (RSFV) and establishing that it belongs to the newly recognized subfamily Spumavirinae (foamy agents) of the RNA virus family Retroviridae. A serological survey of Florida raccoons was carried out to determine the frequency of occurrence and geographical distribution of the virus.

III. MATERIALS AND METHODS

A. Laboratory Animals and Sera

Raccoons used in this study were obtained from the Florida Game and Fresh Water Fish Commission and the National Wildlife Refuge Service and from a local supplier. They were caged individually.

Serum samples were obtained from anesthetized animals by jugular venipuncture using sterile disposable syringes or Vacutainer needles and tubes. The blood was then transferred to plastic centrifuge tubes and allowed to clot. The clot was then reamed from the walls of the tube with a glass rod and left to recede overnight at 4°C. The serum was collected after centrifugation at 2000 rpm for 20 minutes in a refrigerated centrifuge.

Raccoons which were to be euthanatized for microbiologic examination of their tissues were anesthetized with an appropriate dose of ketamine hydrochloride. Death by exsanguination was then achieved by cardiac puncture, generally by use of a sterile 30 ml syringe and 18 gauge 1½ inch sterile disposable needle. After careful and

asceptic opening of the carcass, various organ and tissue samples were placed in sterile disposable petri dishes for transport to the tissue culture facility. Swab samples of various body orifices were also taken for virus isolation attempts.

Rabbits were obtained through the facilities of the College of Veterinary Medicine. Three animals were experimentally infected with the raccoon virus. Pre-inoculation and subsequent blood samples were obtained by stylet puncture of a marginal ear vein. Serum was obtained as described above. Rabbit anti-RSFV serum was used in indirect immunofluorescence tests with fluorescein-labeled goat anti-rabbit globulin serum.

Raccoon sera collected from throughout the state of Florida were made available by Dr. William Bigler of the Florida Department of Health and Rehabilitative Services in Tallahassee, Florida.

B. Cells and Media

Cell cultures used in this study include the established cell lines Statens Seruminstitut Rabbit Cornea (SIRC), Baby Hamster Kidney (BHK) and Crandell Feline Kidney (CrFK). In addition, primary cultures of rabbit kidney

and kitten lung cells were prepared in the laboratory using standard trypsin digestion methods. For storage, heavy suspensions of these cells in growth medium with 10% dimethyl sulfoxide were dispensed into individual 1 ml glass ampoules and flame-sealed. The ampoules were then placed in alcohol bath and cooled to 4°C for twenty minutes. They were transferred to a -20°C freezer for thirty minutes and then to a -70°C freezer for one hour. After this time, the ampoules were removed from the alcohol bath and stored in liquid nitrogen for subsequent use.

Eagle's Minimal Essential Medium supplemented with 10% fetal calf serum and 0.5% lactalbumin hydrolysate was used for growing cell cultures. Penicillin (100 units/ml) and streptomycin (100 ug/ml) were also added to the medium.

C. Virus Isolation

1. Direct cell cultures: Aseptically collected tissue samples were finely minced, washed with isotonic phosphate buffered saline and subjected to .25% trypsin digestion for 45 minutes while being magnetically stirred. The tissue digests were filtered through gauze-wrapped funnels to get rid of larger pieces and

centrifuged for 15 minutes at 900 gs. The supernatants were discarded and the pellets resuspended in growth media. The cell suspensions were dispensed into culture flasks.

Cultures initiated in that manner were serially transferred at least three times as soon as they would become confluent. Passage was accomplished by trypsin-versene dispersion of the cells, resuspension into fresh culture media and seeding into new culture flasks.

With each transfer, companion Leighton tube cultures were initiated. After the cells had grown-in the cover slips were removed and subjected either to Giemsa staining or indirect immunofluorescent staining. For Giemsa staining, the cover slips were placed on racks, rinsed with isotonic phosphate buffered saline, fixed for 10 minutes in methyl alcohol, stained with 10% Giemsa for 20 minutes, dehydrated in acetone and xylene, and mounted on glass slides for microscopic examination. Indirect immunofluorescence techniques are detailed in a later section (III, F, 2). Cover slip preparations were found to be the best way to monitor infection and cytopathic effect of the virus.

2. Tissue suspensions: Portions of each tissue collected were minced and ground with Ten Broeck tissue grinders. The supernates were decanted, centrifuged to get rid of debris and stored at -70°C. For assay of viral content, 1 milliliter of the preparations was inoculated into growing cultures of SIRC or BHK cells. Such cultures were passed at least three times and accompanying Leighton tube cultures were prepared. After the cells grew to confluency, the cover slips were removed and stained as described above.

3. Inoculation of buffy coat cells into established cell lines: Blood collected aseptically into 10 ml syringes was quickly ejected into flasks and swirled to promote defibrination and prevent clotting. The defibrinated blood was centrifuged at 2000 rpm for 10 minutes. Pasteur pipettes were used to aspirate buffy coat cells from the top of the pellet. These were immediately inoculated into growing cultures of SIRC or BHK cells. After 24 hours at 37°C, the growth media were decanted and the monolayers washed. Fresh growth fluid was then added. After reaching confluency, cultures were passed with companion Leighton tubes and monitored for infection as described above.

4. Swab samples: Two milliliters of growth medium were dispensed into sterile screw-cap tubes. Cotton swabs on wooden applicator sticks were used. Prior to obtaining a sample, a sterile swab was moistened with medium and then used to vigorously swab the appropriate area: throat, nasal, rectal or vaginal area. The swab was then placed in one of the tubes containing the growth medium, and the applicator stick broken off the wall of the tube. The samples were stored at -70°C until virus recovery was attempted.

To check for viral content, each sample was filtered through a .3 micron filter and a .2 ml volume inoculated into growing cultures of BHK or SIRC cells. The cultures were passed at least three times with companion Leighton tube. The presence of virus was checked by Giemsa or indirect immunofluorescence as previously described.

D. Stock Virus Preparation

Two virus stocks were used, one grown in BHK cells and one grown in SIRC cells. The stocks were prepared by inoculating .5 ml of virus isolate into cultures in which the cells had not reached confluency. After the cells had grown in, blind passages were performed by subcultivation

of the cultures. Companion Leighton tube cultures were initiated with each transfer. When the cells were grown in, the cover slips were removed and stained by immunofluorescence. When 60% or more of the cells became infected, usually after 2 or 3 transfers, the cultures were frozen, thawed and centrifuged at 2000 rpm for twenty minutes. Freeze-thawing was done to release some of the cell-associated viruses. The supernatant growth medium containing cell-free virus constituted the viral stock preparation and was conserved at -70°C.

E. Viral Procedures

1. Plaque assay: The method of Parks and Todaro (25) was followed. BHK cells were seeded into 50 mm petri dishes. After 24-48 hours, before the cells reached confluency, the drained monolayers were inoculated with the appropriate viral dilutions. Each dilution was done in quadruplicates. The virus was allowed to adsorb for a 2 hour period at 37°C; fluid medium was then replaced. After a further 24 to 48 hours incubation, 1% agar in growth medium was applied as an overlay. Seven days later, a second overlay with a 1 to 10,000 dilution of neutral red was added and after another 24 hours in the

dark, plaques were counted using indirect lighting to enhance visualization.

2. Immunofluorescent assay: BHK cells were seeded into Leighton tubes and inoculated with doubling dilutions of virus preparation. The first dilution was a ten-fold dilution which was serially doubled, usually up to a dilution of one in 2560. Four Leighton tube cultures were used for each dilution. After a five-day incubation period at 37°C, the cover slips were removed and subjected to the indirect immunofluorescence procedure using rabbit anti-serum to the virus and fluorescein isothiocyanate (FITC) labeled goat anti-rabbit immunoglobulin serum. The last dilution at which specific fluorescent staining was observed was considered the end-point titer. The titers were calculated according to the appropriate dilution and expressed as fluorescent Focus-Forming Units (FFU) (10).

F. Serologic Methods

1. Immunodiffusion

a. Antigen preparations: BHK cells were grown in one liter bottle in a roller apparatus. One hundred milliliters of medium were sufficient to grow the cells to

confluency when the bottles were rotated at .5 revolutions per minute. With the roller method, greater quantities of cells could be grown more economically than with culture flasks. The bottles were inoculated at an early stage with 2 milliliters of viral stock. When 60% or more of the monolayer consisted of syncytia (usually after two passages by subcultivation) the cells were scraped from the bottle wall with a rubber policeman and centrifuged (900 gs for 20 minutes) in the medium in which they were grown. The supernatant fluid was decanted, the centrifuge tubes drained and the cells resuspended in the small volume of medium still remaining. The concentrated suspension of infected cells was frozen and thawed several times and then centrifuged again at 900 gs for 20 minutes. The opalescent supernatant material constituted the precipitating antigen. It was stored at -20°C until needed. Each liter bottle gave about .4 ml of antigen.

Antigen was also prepared by direct viral concentration. One hundred milliliters of viral stock preparation were centrifuged at 25,000 rpm for 3 hours to pellet the virus. The viral pellet was then resuspended in .2 milliliters of TNE buffer (.01 M Tris-HCl, .1 M NaCl, .005 M EDTA). Some viral preparations were treated with

chloroform prior to concentration in order to eliminate envelope structures from the antigen preparation.

b. Method: Immunodiffusion tests were carried out in a micro-diffusion system in order to conserve antigen. The method developed by Crowle (6) using plexiglass templates to hold the reagents was used.

One per cent Noble agar dissolved in borate buffer (pH 8.5) with 1:10,000 merthiolate was used to fill the space between a plexiglass block and a glass slide. The space was created by supporting the block on each side by three layers of electrical tape. The layer of agar thus formed was about 1 mm thick. After allowing the agar to solidify, the block was removed and replaced by a template. The wells were filled (.05 ml) using 1 ml syringes with 22 gauge needles.

The slides were then held at room temperature for 36-72 hours in a humidified atmosphere created by putting them in glass petri dishes containing wet filter paper. At the end of the time, the templates were carefully removed and the precipitin lines read. The preparations were then soaked in PBS for 24 hours and then in distilled water for another 24 hours to remove unreacted reagents.

They could then be photographed by indirect lighting or dried and stained with Buffalo black solution for permanent records.

c. Antisera: The antisera used for precipitation were those of raccoons and experimentally infected rabbits. Usually the antigen was placed in the center well and the sera to be checked in the surrounding wells. Templates with one central well and six or four surrounding wells were used.

2. Immunofluorescence

a. Preparation of FITC-labelled conjugates: Goat anti-rabbit globulin serum was already available in the laboratory. Rabbit anti-raccoon globulin serum was prepared specifically for this study. Thirty milliliters of raccoon serum was precipitated at 50% and 33% saturation with ammonium sulfate and dialysed against PBS (pH 7.5).

A spectrophotometer was used to evaluate the protein content and the preparation was diluted to 1 mg/ml with PBS.

A rabbit was immunized with the preparation. One milliliter emulsified in an equal volume of Freund's

complete adjuvant was injected I.M. in the "hamstring" muscles. Three weeks later it was bled and its serum checked for precipitating activity with raccoon globulin. One week later 1 mg of raccoon globulin preparation was injected I.V., and after another week the rabbit was anesthetized and exsanguinated by cardiac puncture.

Goat anti-rabbit and rabbit anti-raccoon serums were conjugated with FITC in the manner described in Appendix I. The goat-rabbit indirect system was used to detect and quantitate infection in cells grown in Leighton tubes. The rabbit anti-raccoon conjugate was used with known infected cover slips to detect the presence of antibody to RSFV in raccoon serums.

b. Staining procedure: Infected cover slips from Leighton tubes were removed and placed in staining racks. The racks were then rinsed in phosphate-buffered saline and fixed in acetone. The cover slips were allowed to dry and placed on glass slides. With a Pasteur pipette a thin layer of the anti-viral serum was applied on the cover slips. They were then incubated for one hour at 37°C in humidified glass petri dishes. The slips were then removed from the glass slides, placed back in the racks

and washed with 2 changes of stirred PBS. The racks were rinsed in distilled water after which the cover slips were allowed to dry and placed again on glass slides. The FITC labeled antiserum was layered on them. The preparations were again incubated for an hour at 37°C in humidified petri dishes. After this time the cover slips were removed, reinserted in racks and the washing process with PBS and distilled water repeated. They were then dipped 3 times in eriochrome black solution as a counter-stain and rinsed in two changes of distilled water. The backs of the cover slips were dried with tissue paper and they were mounted, cell side down, with 10% buffered saline in glycerine on glass slides. The preparations were then examined for specific fluorescence using dark-field, ultraviolet light microscopy.

3. Neutralization: Sera from raccoons and from experimentally infected rabbits were tested for neutralizing activity. All sera checked for neutralizing antibodies were inactivated for 30 minutes at 56°C. Doubling dilutions of the serum to be tested were made. Each dilution (1 ml) was incubated with an equal amount of viral stock containing 200 FFUs. The mixtures were incubated for 1 hour at 37°C.

Then .2 milliliter of each dilution was added to a set of four Leighton tubes containing 1.8 ml of BHK cell suspension at a concentration of 10^5 cells/ml. The tubes were placed in racks and incubated 5 days at 37°C. At the end of this period, the cover slips were removed and stained by indirect immunofluorescence using the goat-rabbit reagents. The reciprocal of the last dilution at which no specific staining could be seen was considered the titer.

G. Sensitivity to Lipid Solvents

The method of Andrewes and Horstmann (2) was followed. A mixture of 13 parts virus in MEM and 1 part anhydrous ether was kept at 4°C for 16 hours with periodic vigorous shaking. The ether was then decanted, residuum evaporated, and titrations done by immunofluorescence.

The effect of chloroform was tested using 1 part chloroform and 19 parts viral culture (4). The preparation was also incubated for 16 hours at 4°C with intermittent shaking. The virus culture was then decanted and titrated.

H. Viral Replication Studies

1. Effect of BUDR: The method of Parks and Todaro (25) was used. Two growing cultures of BHK cells were inoculated with 2 milliliters of viral stock preparation. After a 1-2

hour adsorption, medium alone or medium with BUDR (10 ug/ml) was added and incubated in the dark. Twenty-four hours later, the culture fluids were removed, the monolayers washed 3 times with media and fresh medium added. At 48 hours post-inoculation, virus was harvested by freezing and thawing the infected cultures. They were then centrifuged at 900 gs for 20 minutes to pellet cell debris. The supernatants were then assayed by the indirect immunofluorescence technique.

2. Uridine uptake and effect of Actinomycin D: The effect of actinomycin D on tritiated uridine uptake was studied as described by Parks et al. (26). BHK cells at advanced stages of infection were incubated for 24 hours with 30 uCi/ml of ^{3}H-thymidine, ^{3}H-uridine alone, and ^{3}H-uridine plus actinomycin D (2 ug/ml). The actinomycin D was added 4 hours prior to the label. The cultures were freeze-thawed; the supernatant fluids were clarified by centrifugation (900 gs, 20 minutes) and precipitated with saturated ammonium sulfate. The precipitate was redissolved 1.5 ml of TNE buffer and .2 ml of this preparation was layered on top a 15-55% sucrose gradient and centrifuged at 200,000 g for 2 hours. Collected fractions were counted in scintillation fluid in a liquid scintillation counter.

I. Reverse Transcriptase Assay

Reverse transcriptase assays were performed on virus preparations concentrated one hundred fold. Two hundred milliliters of virus stock were centrifuged at 75,000 g for 3 hours. The pellet was then resuspended in 2 milliliters of TNE buffer. The assays were performed under conditions described by Parks et al. (26). The viral concentrate was filtered through a .3 micron filter and varying amounts (10, 20, 30 and 50 lambdas) were incubated for 2 hours with 50 lambdas of a reaction mixture containing 40 mM tris HCl, 60 mM KCl, 2 mM $MnCl_2$, 2 mM of dithiothreitol, 20 uM of ^{3}H-TTP, 5 mM of each dATP, dCTP, dGTP, and .2% triton X-100. To each reaction, 1.25×10^{-3} mg of the synthetic nucleic acid template rA:dT was added. After incubation, .1 ml of "carrier" salmon sperm DNA (2.5 mg/ml), .1 ml of .1 M PP_i and .5 ml of cold 3.5% perchloric acid-0.35% uranyl acetate were added per .1 ml of reaction mixture. After 10 minutes at 0^o, the resulting precipitate was collected on a Whatman glass filter (GF/C 2.4 cm) and washed 3 times (10 ml portions) with cold 1 N HCl, 3 times with 1 N HCl-0.1 M PP_i and once with absolute ethanol. The filter was dried and its radioactivity determined. The absolute requirement for manganese was checked by carrying out the reactions with magnesium ion instead.

The effect of decreasing Triton X-100 concentration to .02% was also determined. The absolute requirement for manganese was checked by carrying out the reactions with magnesium ion instead. The effect of decreasing Triton X-100 concentration to .02% was also determined.

Viral concentrate was layered on top of a 15-60% sucrose gradient and centrifuged at 200,000 gs for 2 hours. Fractions were collected by bottom puncture and assayed directly for polymerase activity. The per cent sucrose of each fraction was read in a refractometer and the relative buoyant densities determined.

J. Electron Microscopy

Infected SIRC cells were examined. The cells were fixed with 3% buffered glutaraldehyde and 1% osmium tetroxide. The preparations were then dehydrated stepwise in 50, 70, 80, 95 and 100% ethanol and finally with absolute propylene oxide. The resulting tissues were embedded in epoxy resin in gelatin capsules which were cured for 3 days at 45°C.

Sections of tissue 200 to 500 Å thick were cut from the resin using a Porter Blum ultramicrotome manufactured by Ivan Sorvall, Inc. The sections were placed on grids, stained with uranyl acetate and lead citrate, and viewed.

IV. RESULTS

A. Virus Isolation

The raccoon syncytium-forming virus (RSFV) was first isolated from various organs of raccoons captured on Marco Island, Collier County. Four animals were received from the Florida Game and Fresh Water Fish Commission. They were euthanatized by exsanguination via cardiac puncture and their sera collected. Primary cell cultures were derived from spleen, kidney, lung and greater omentum. The cells grew normally to confluency in the tissue culture flasks without any noticeable abnormalities.

After 2 to 3 passages by subcultivation definite cytopathic effect manifested by syncytia formation became evident in the organ cultures of 3 of the 4 raccoons. Affected cells became rounded and more refractile in appearance by light microscopy. Simultaneously with these changes, vacuoles developed in the cytoplasms of these cells giving a foamy appearance under low powered magnification. In affected areas, cells lost their regular orientation in the monolayer. These changes were presumed

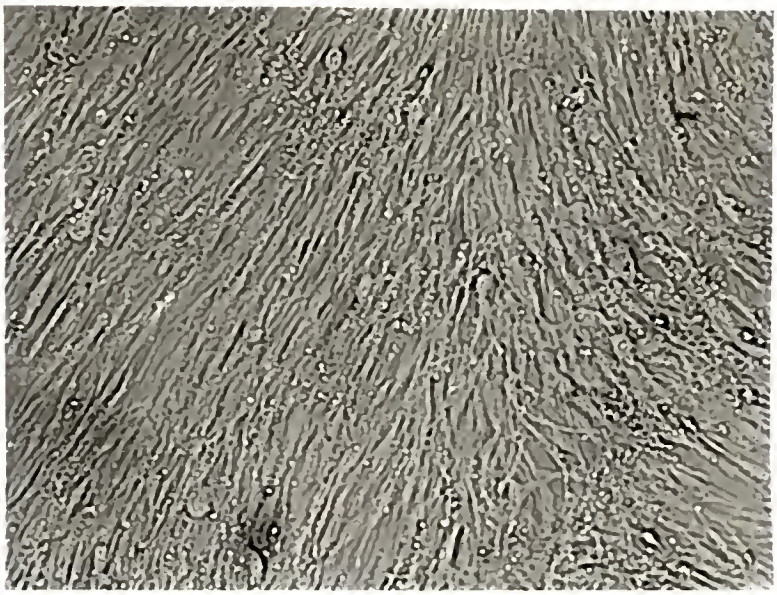
to be due to the presence of a latent virus. Its cytopathic effect closely paralleled that of the syncytium-forming viruses of simian, bovine and feline origins.

To confirm this hypothesis, cells from cultures which exhibited these manifestations were co-cultured with the established cell lines SIRC and BHK. After one subcultivation, syncytia formation appeared. Upon observation over a period of time, progressive involvement of cells in syncytia formation could be seen. Further passage resulted in a sharp increase of fused and vacuolated areas (see Figs. 1, 2 and 3). More nuclei could be seen accumulating into syncytia through a process of incorporation of adjacent cells. With longer incubation, some of the large multinucleated cells would become detached from the bottom of the culture flask leaving irregularly shaped microscopic plaques in the monolayer. Infected cultures could not be transferred beyond third or fourth passage since this resulted in total cell destruction.

Companion Leighton tube cultures were initiated at the time of each transfer. After the cells were grown in, the cover slips were removed and stained with 10% Giemsa (see Figs. 4 and 5). Syncytia with varying degree of multinucleation could be seen in these stained preparations

Fig. 1. Uninfected BHK cells grown in MEM supplemented with 10% calf serum (Magnification X100).

Fig. 2. Second passage BHK cells inoculated with RSFV. Large refractile giant cells can be seen along with an area of cell lysis. Cells have lost their regular orientation in the monolayer.



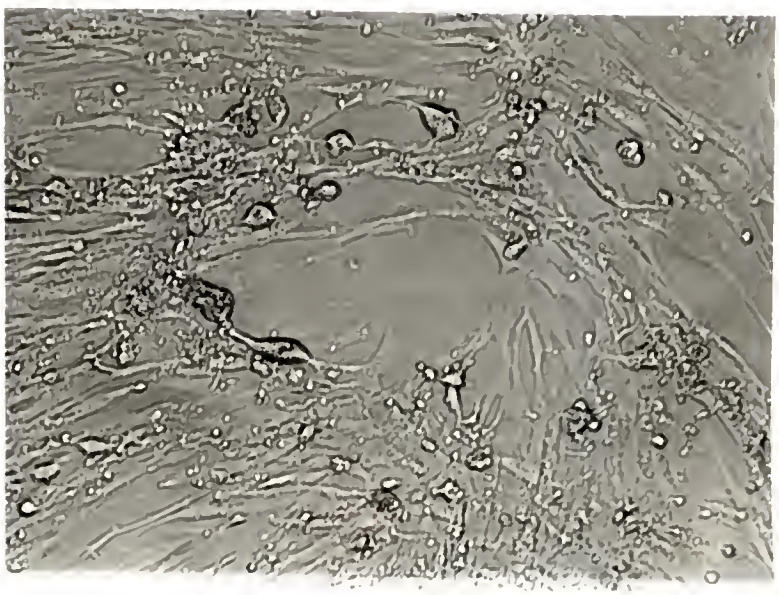
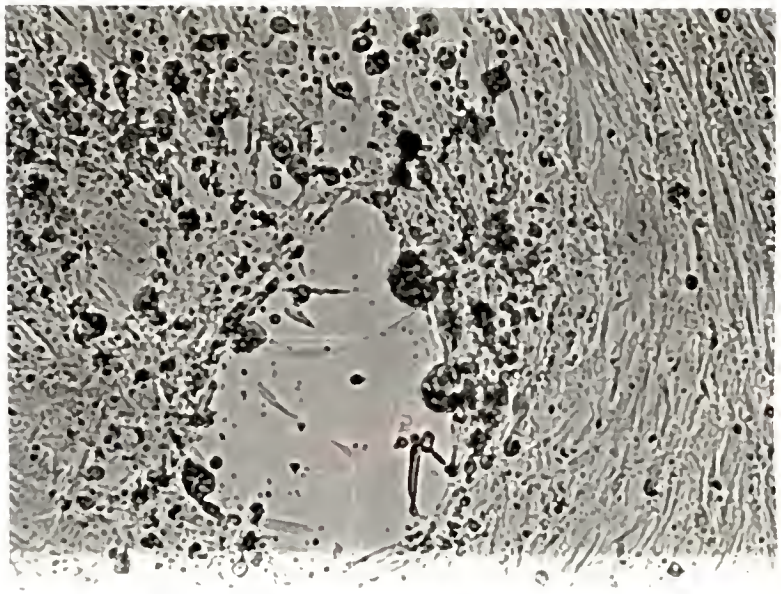
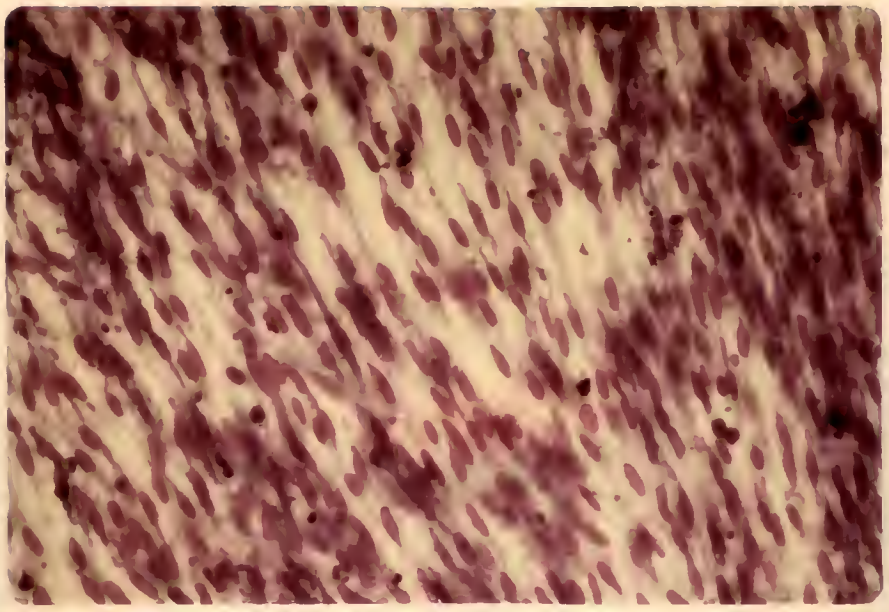


Fig. 3. RSFV-infected BHK cells. Irregularly shaped microscopic plaque surrounded by large syncytial structures that have rounded up and are about to become detached from the bottom of the culture dish.

Fig. 4. BHK cells grown in a Leighton tube. The cover slip was removed, fixed and stained with 10% Giemsa.



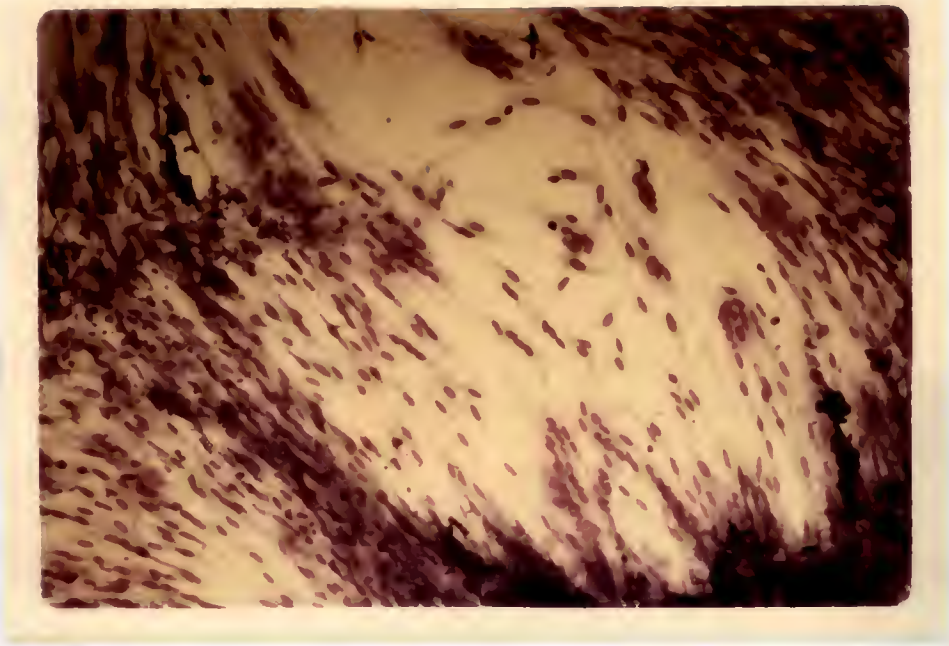


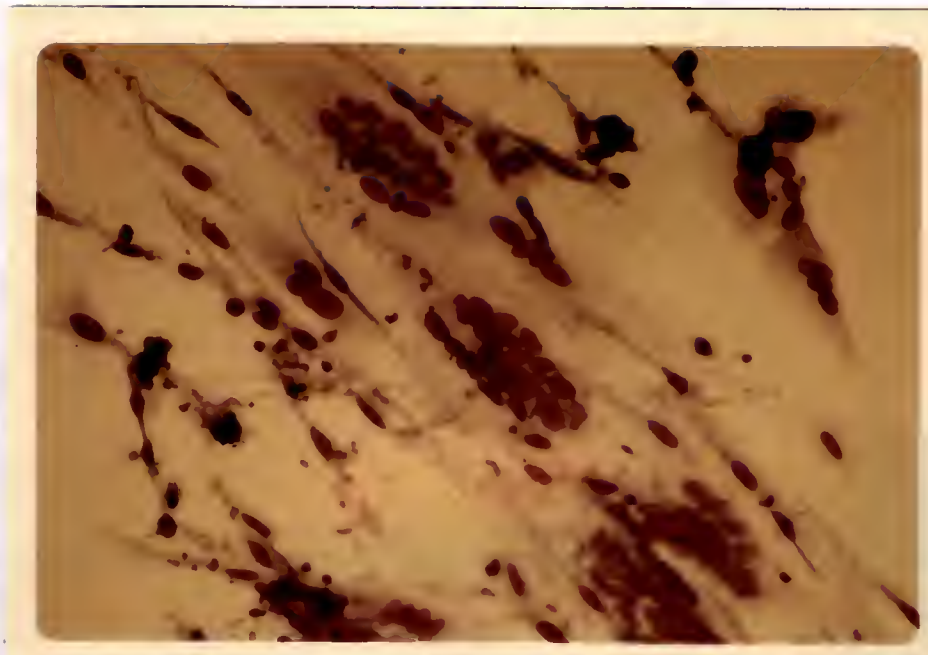
(see Fig. 6). The nuclei involved could be seen evenly distributed in the cytoplasm, clustered in certain areas, or in ring formation around the peripheral cytoplasm. Since a characteristic host response to infection by syncytium-forming viruses is the production of precipitating antibodies, viral antigen was extracted from infected cells to check various sera for precipitating activity. A concentrated lysate of infected cells constituted the precipitating antigen. In order to conserve antigen tests were performed employing a microsystem developed by Crowle (6). The sera of animals from which the virus could be isolated had precipitating activity with the antigen. In contrast no virus could be isolated from blood or any of the organs of raccoons whose sera showed no line of precipitation (see Fig. 7). Thus, a procedure was developed by which raccoons could be quickly screened for infection with RSFV.

Persistent infection of a rabbit with RSFV was produced by administering infected SIRC cells intravenously. A preinoculation bleed showed that the animal's serum contained no activity against the viral antigen. A month later a second bleed was performed and precipitating antibodies were demonstrated in the serum. Precipitating

Fig. 5. RSFV infected BHK cells after second passage (Magnification X100). Cells were grown in a Leighton tube and the cover slip was stained with 10% Giemsa.

Fig. 6. RSFV-induced syncytia in infected BHK cells. Varying degrees of multinucleation can be seen in giant cells (Magnification X200).





activity persisted for over a year (see Fig. 8). Peripheral blood leukocytes (buffy coat) of the rabbit were inoculated into growing SIRC cell cultures. The latter developed characteristic CPE after subcultivation and totally degenerated upon further passage. Furthermore, antigen extracted from SIRC cells infected with the rabbit buffy coat precipitated with positive raccoon sera, demonstrating the animal to be persistently infected with RSFV.

Two raccoons were received from the National Wildlife Refuge on Merritt Island. Both were bled and their sera checked for antibodies to RSFV by immunodiffusion and immunofluorescence and both were negative. Throat, nasal, rectal and vaginal swabs and buffy coat cells from each raccoon were inoculated into growing cultures of SIRC cells. After 3 passages, the cover slips from companion Leighton tubes were stained by immunofluorescence. All were negative. The raccoons were killed by exsanguination and primary cultures were initiated from spleen, omentum, kidney and lungs. After 3 passages no CPE was observable and immunofluorescence performed on companion cover slips proved negative. Also, suspensions from each of the organs were inoculated into SIRC cells with no effect after 3 passages. These experiments reinforced prior observation that the

Fig. 7. Immunodiffusion using antigen extracted from infected cells. The antigen was placed in the center well and various raccoon sera in the six surrounding wells.
Sera 1, 2 and 4 gave 2 lines of precipitation
Serum 5 produced one band
Sera 3 and 6 did not react

Fig. 8. Detection of precipitating antibodies to RSFV antigen in rabbit infected by injection of infected SIRC cells intravenously. The rabbit serum was placed in well 1 and shows a line of identity with positive raccoon sera in wells 2 and 6. The preparation was stained with Buffalo black.


2
3
1
4
6
5


4
5

Fig. 9. Immunodiffusion with untreated and chloroform treated antigen. Positive raccoon serum (75-1) was placed in the center well.
1--Antigen prepared from infected BHK cells
2--Concentrated virus
3--1 treated with chloroform
4--Concentrated virus preparation from viral stock preparation treated with chloroform prior to concentration.

Fig. 10. Immunodiffusion with antigen prepared from infected BHK cells.
1--Rabbit injected with infected SIRC cells
2--Rabbit receiving 2 ml of cell-free virus stock prepared from infected BHK cells
3--Raccoon serum 75-1
4--Same as 2
5--Same as 3
6--Rabbit infected by injection of 5 ml of whole blood from rabbit 1.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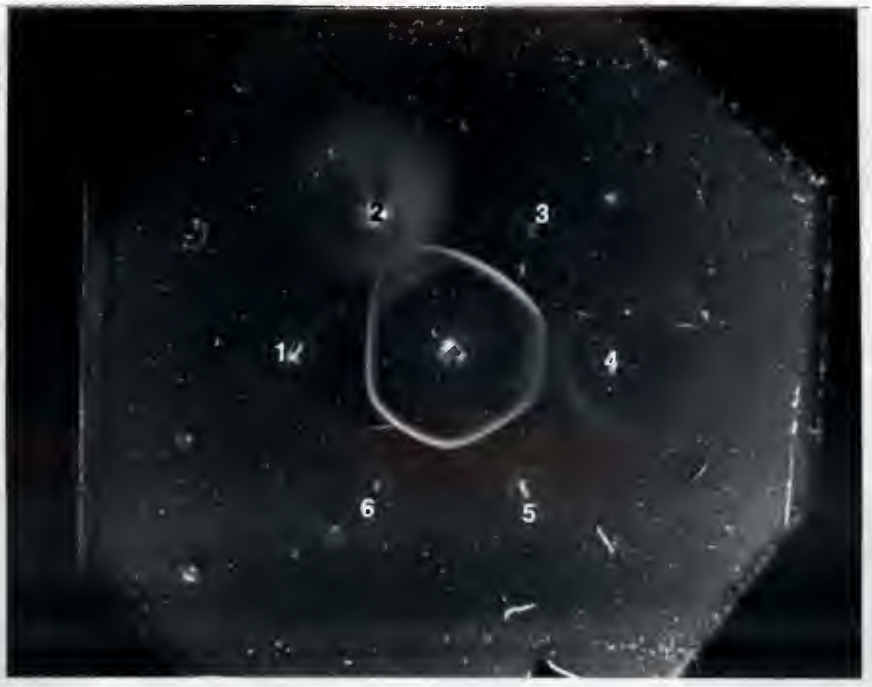
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presence of precipitating antibodies in serum could be used as a presumptive test for viral infection.

Table II shows tissues from which virus was recovered.

Table II

Tissues from Which RSFV Was Recovered

Spleen
Greater omentum
Kidney
Lungs
Peripheral blood leukocytes

B. Antigen Preparation

As mentioned above, RSFV antigen for immunodiffusion was prepared by freeze-thawing very heavy suspensions of infected cells and collecting the lysate. This is the method described by Malmquist et al. (24) with the bovine agent and Gaskin and Gillespie (12) with FeSFV. Treating the RSFV antigen with chloroform did not destroy its precipitating property (see Fig. 9). This suggests that there is no lipid in the antigenic sites involved.

Attempts were made to prepare RSFV antigen by concentrating viral stock preparations. Five hundred-fold concentrations were made by ultracentrifugation. Viral core concentrates were also prepared by treating virus stock with chloroform prior to ultracentrifugation. Neither viral nor chloroform treated viral concentrates precipitated with RSFV antisera (see Fig. 9).

The number of precipitation lines seen in gel diffusion depended both on the serum and the antigen preparation. Some sera produced two precipitin lines and others only one with the same antigen. When two bands were seen, one was usually quite pronounced and the other weak and diffuse. The major band was in identity with that of sera producing only one line of precipitation. With some antigen preparations, however, only the major band was produced no matter what antisera were used. The same phenomenon was observed by Malmquist et al. (24) who developed the system for the bovine agent. This was presumed to be due to a concentration effect.

C. Experimental Infection

As mentioned in section A, chronic infection of a rabbit was achieved by administering infected SIRC cells

intravenously. Serum precipitating antibodies have persisted for over a year and virus could be routinely recovered from its peripheral blood leukocytes.

When 5 milliliters of whole blood from the infected rabbit was injected into a marginal ear vein of a second rabbit, circulating antibodies to RSFV antigen could be detected five weeks later. By immunodiffusion, lines of identity with the donor rabbit serum and positive raccoon serum could be seen (see Fig. 10).

A third rabbit was infected with 2 milliliters of cell-free viral stock preparation (grown in BHK cells) which was injected intravenously. A month later, its serum showed a very weak line of precipitation against RSFV antigen. This line was in identity with precipitin lines formed by other anti-RSFV sera (see Fig. 10). Another precipitation band was also observed. Since the virus stock was prepared by freeze-thawing BHK cultures to release intracellular virus, the preparation also contained cellular and medium components. Therefore, the rabbit's serum was tested against antigen prepared with uninfected cells and formed a precipitin band indicative that the rabbit had responded to at least one antigen of non-viral origin.

Twenty-five days after the first bleed, a second serum sample was taken. This sample no longer had precipitating activity against BHK antigen but gave a strong line of identity against RSFV antigen when tested with other positive sera (see Fig. 11). The persistence of such antibodies is indicative of chronic infection. Therefore, it was found that infection could be initiated with infected cells, whole blood from an infected donor and with cell-free virus.

D. In Vitro Host Range

The syncytium-forming viruses replicate and induce CPE in numerous cell lines from a variety of mammalian hosts. The viruses productively infect epithelial and fibroblastic cells. The raccoon isolate was shown to infect primary cell cultures of rabbit kidney and kitten lung and the established cell lines SIRC, BHK and CrFK.

In each cell culture system, it was necessary to inoculate growing cultures in order to observe infection. No CPE could be readily detected in the monolayers until they were subcultivated. Kitten lung and BHK cells proved to be more susceptible to viral induced CPE than the others since syncytia and areas of cellular destruction appeared

more rapidly. The infected kitten lung cells rarely survived a second transfer. None of the infected cells tested could be transferred beyond 4 passages.

With each subcultivation, companion Leighton tube cultures were initiated. When the cells were grown in, the cover slips were removed and stained by indirect immunofluorescence using rabbit immune serum and FITC labeled goat anti-rabbit globulin serum. Large syncytia with intense nuclear staining could be seen (see Figs. 13 and 14). The reddish appearance of control cells is due to the eriochrome black counterstain (see Fig. 12). There was a tendency of nuclei involved in giant cell formation to arrange themselves uniformly around the periphery of the cytoplasm. Table III shows that all cells showing foamy degeneration exhibited viral antigens detectable by immunofluorescence.

Table III

Host Range of RSFV

Cell Types Tested	Indirect Immunofluorescence	CPE
SIRC	+	+
BHK	+	++
RK	+	+
CrFK	+	+
KL	+	+++

Fig. 11. Immunodifussion showing line of identity produced by the sera of three rabbits, each infected by a different method as indicated in Fig. 10.

Fig. 12. Indirect immunofluorescence on uninfected BHK cells. The red appearance is due to the eriochrome black counterstain.


2
3
1

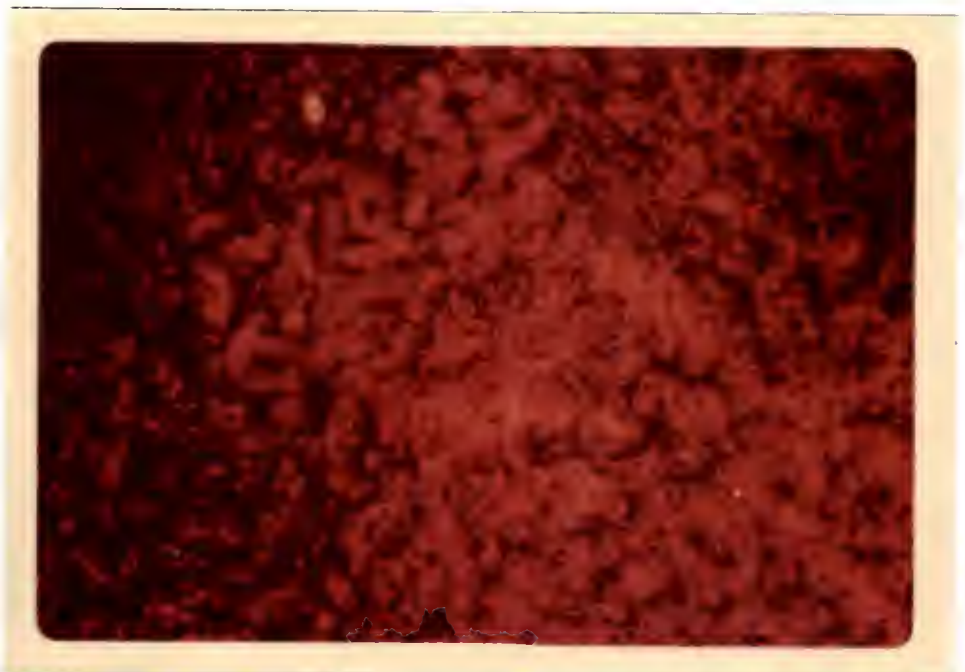
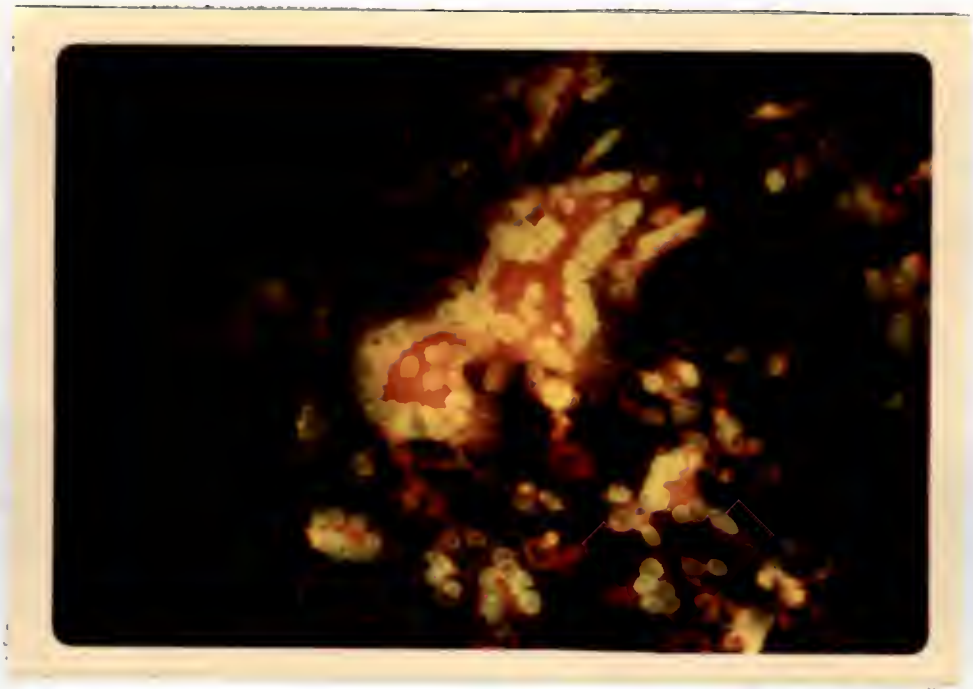
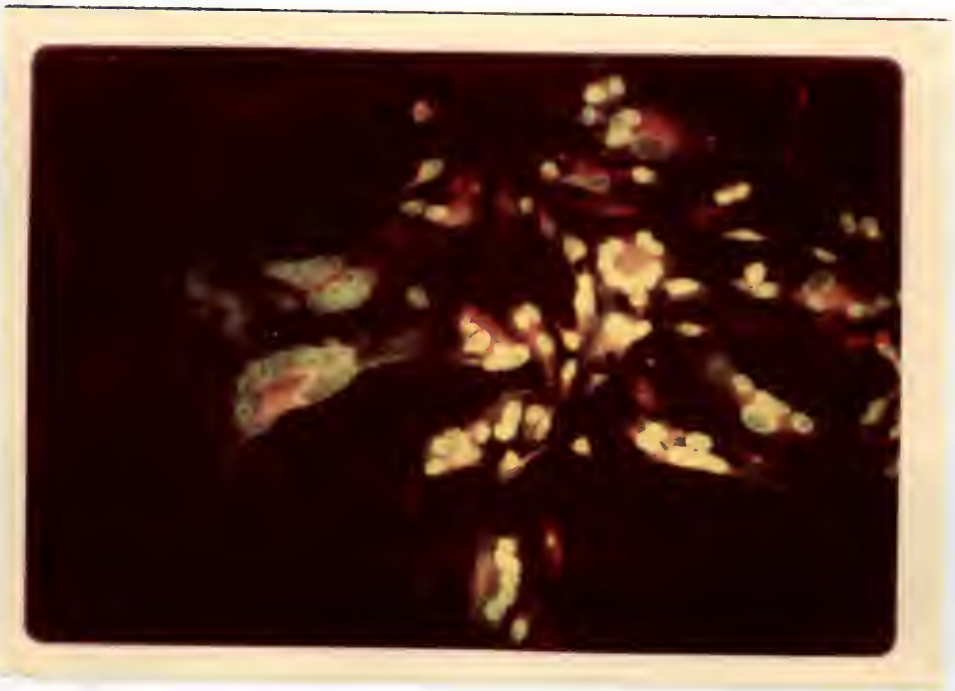


Fig. 13. Indirect immunofluorescence on infected SIRC cells. Rabbit antiserum to RSFV and FITC-labeled goat anti-rabbit globulin serum were used to stain Leighton-tube cover slips. Syncytia with varying degree of multinucleation can be seen. Note the intense nuclear staining.

Fig. 14. Indirect immunofluorescent staining of RSFV infected primary rabbit kidney cell culture. Nuclei in ring formation around the periphery of the cytoplasm can be seen.





E. Cytopathology and Assay of RSFV

Parks and Todaro (25) have pointed out that the lack of adequate quantitative assay procedures has hindered the study of the foamy agents. They described a plaque assay system which proved successful with simian foamy virus Types I and III but not with the bovine agent. This method consisted of the application of a 1% agar overlay 24 to 48 hours after inoculation of the virus into 50-mm petri dishes. Seven days later a second overlay with a 1 to 10,000 dilution of neutral red was added and plaques counted 24 hours later. This method did not succeed with RSFV. A .3% ion-agar overlay, followed by removal of the agar 7 days later and staining with crystal violet was also unsuccessful.

The problem was similar to that encountered by Parks and Todaro (25) with the bovine agent in that most of the plaques that did form were not large enough to be counted microscopically and not all foci of infection developed plaques. By performing titrations in Leighton tubes and then staining the cover slips with Giemsa or by indirect immunofluorescence, an appraisal was made of the cytopathic properties of RSFV. The number of macroscopically visible

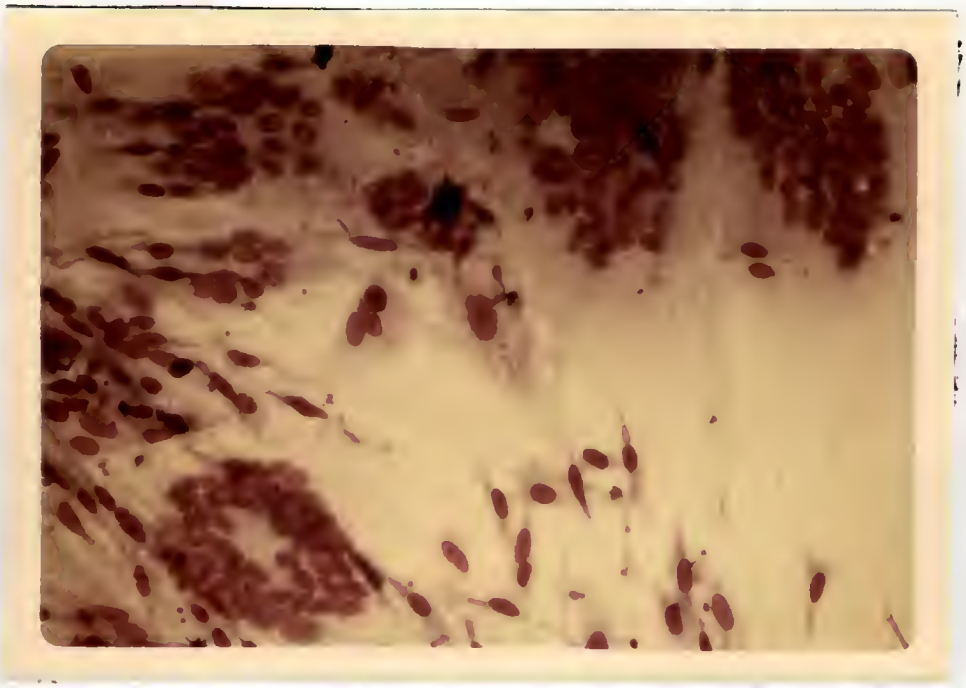
plaques was less than 50% of the plaques that could be detected microscopically (see Fig. 15). In addition, the total number of plaques did not accurately reflect the number of infectious units inoculated into the system. Giemsa staining demonstrated many syncytia where no plaque was apparent. That these syncytia were of viral origin was confirmed by immunofluorescent staining (see Fig. 16). Immunofluorescence also showed another characteristic of RSFV: cells containing viral antigens were not always involved in syncytia formation or any other manifestations of CPE (see Fig. 17).

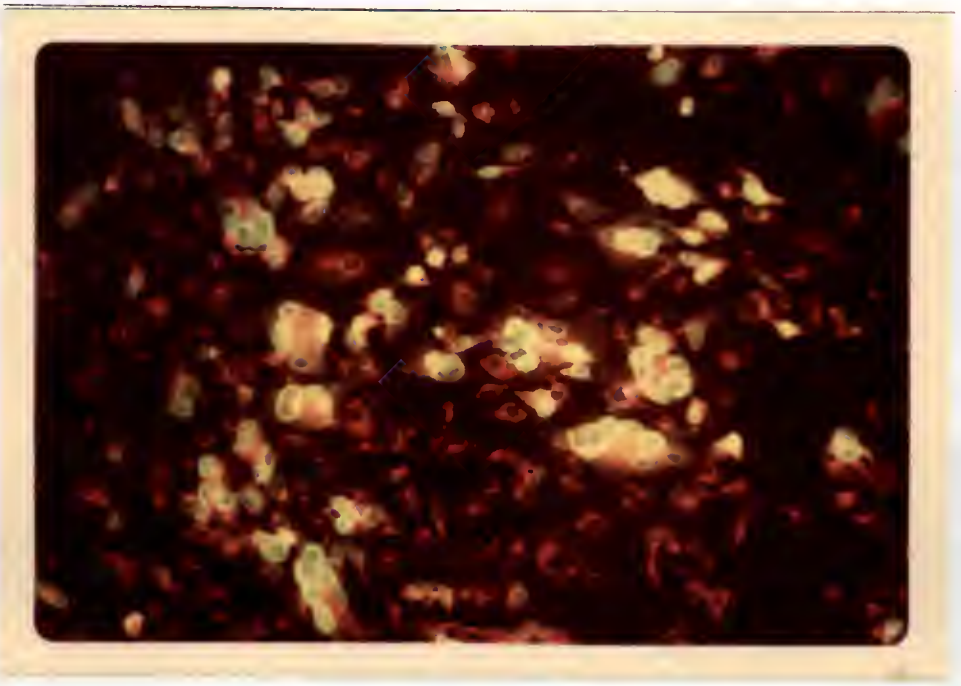
Some simian foamy viruses have been reported to induce a carrier state in certain cell lines (5). This was attempted with RSFV, but after repeated passages (usually 2 to 4) all cells tested were ultimately destroyed.

It was found that the most accurate way to quantitate the virus was by an immunofluorescent end-point assay. Different preparations of viral stocks (prepared by freeze-thawing infected cultures to release cell-associated virus and collecting the supernatants) were found to contain from .8 to 3.2 x 10^3 fluorescent Focus-Forming Units (FFU) per ml.

Fig. 15. Microscopic plaque surrounded by large multinucleated cells as seen after Giemsa staining of BHK.

Fig. 16. Indirect immunofluorescent staining of infected cover slip showing many syncytia with no area of cell lysis.





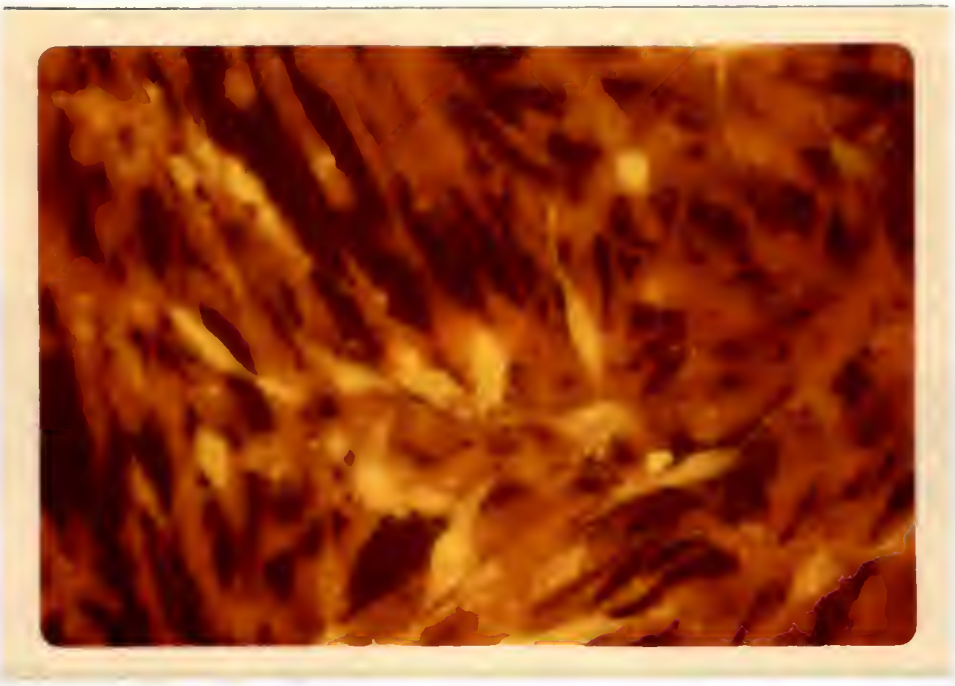


Fig. 17. Indirect immunofluorescence revealing infected cells without any apparent cytopathic effect. In this preparation the infected BHK cells show nuclear and cytoplasmic staining.

F. Viral Neutralization

Neutralizing antibodies have been found in sera of hosts infected by the foamy agents. Malmquist et al. (24) and Gaskin and Gillespie (12) have shown that precipitating antibodies were indicative of chronic infection with the bovine and feline agents. Gaskin (11) has also shown precipitating antibodies could be demonstrated in the absence of neutralizing antibodies.

Neutralization tests were carried out with five raccoon sera that were checked for precipitating activity. Indirect immunofluorescence on infected cover slips using rabbit anti-raccoon globulin conjugate was also carried out to detect the presence of anti-RSFV antibodies. Table IV shows the result of these experiments. They confirm that the precipitin test is a very reliable method to screen sera for infection with RSFV.

G. Sensitivity to Lipid Solvents

As with all other enveloped viruses the syncytium-forming viruses are sensitive to lipid solvents such as ether and chloroform. As indicated in Table V, treatment of the raccoon syncytium-forming virus with ether and

Table IV

Antibodies to RSFV as Determined
by Three Methods

Raccoon Number	Neutralization Titer	Immunodiffusion	Indirect Immunofluorescence
G-75-1	32	+	+
G-431	128	+	+
G-556	16	+	+
70F-2	-	+	+
LR-5-77-1	-	-	-
DC-4 (Rabbit)	64	+	+

chloroform resulted in total inactivation, indicating the destruction of essential lipids in the viral envelope. Since the integrity of the envelope seemed to be essential for initiating infection, it can be assumed that special attachment sites on the envelope are necessary for viral entry into target cells.

H. Viral Replication Studies

1. Effect of BUDR: Low concentrations of bromodeoxyuridine in culture fluids have been shown to markedly inhibit replication of the foamy agents (25). In contrast, iododeoxyuridine has been shown to have much less of an effect. Parks and Todaro (25) have suggested that since the brominated derivative of uridine structurally resembles thymidine more closely than the iodonated derivative, it could be that the reverse transcriptase of the syncytium-forming viruses is able to discriminate between the two halogenated derivatives of thymidine.

The incorporation of 10 ug/ml of BUDR in growth media completely inhibited the replication of the RSFV. Controls grown under identical conditions without BUDR gave substantial yields. Table V shows the results of the experiment.

Table V

Effect of BUDR on RSFV Replication; Also Shows Inactivation of the Virus by Lipid Solvents

Virus	Titer
RSFV Control	3.2×10^3 FFU*
RSFV Grown in BUDR	None detectable
RSFV treated with Chloroform	None detectable
RSFV treated with Ether	None detectable
RSFV maintained** 16 hours at 4^oC	$.8 \times 10^3$ FFU

*Titer expressed as fluorescent Focus-Forming Units

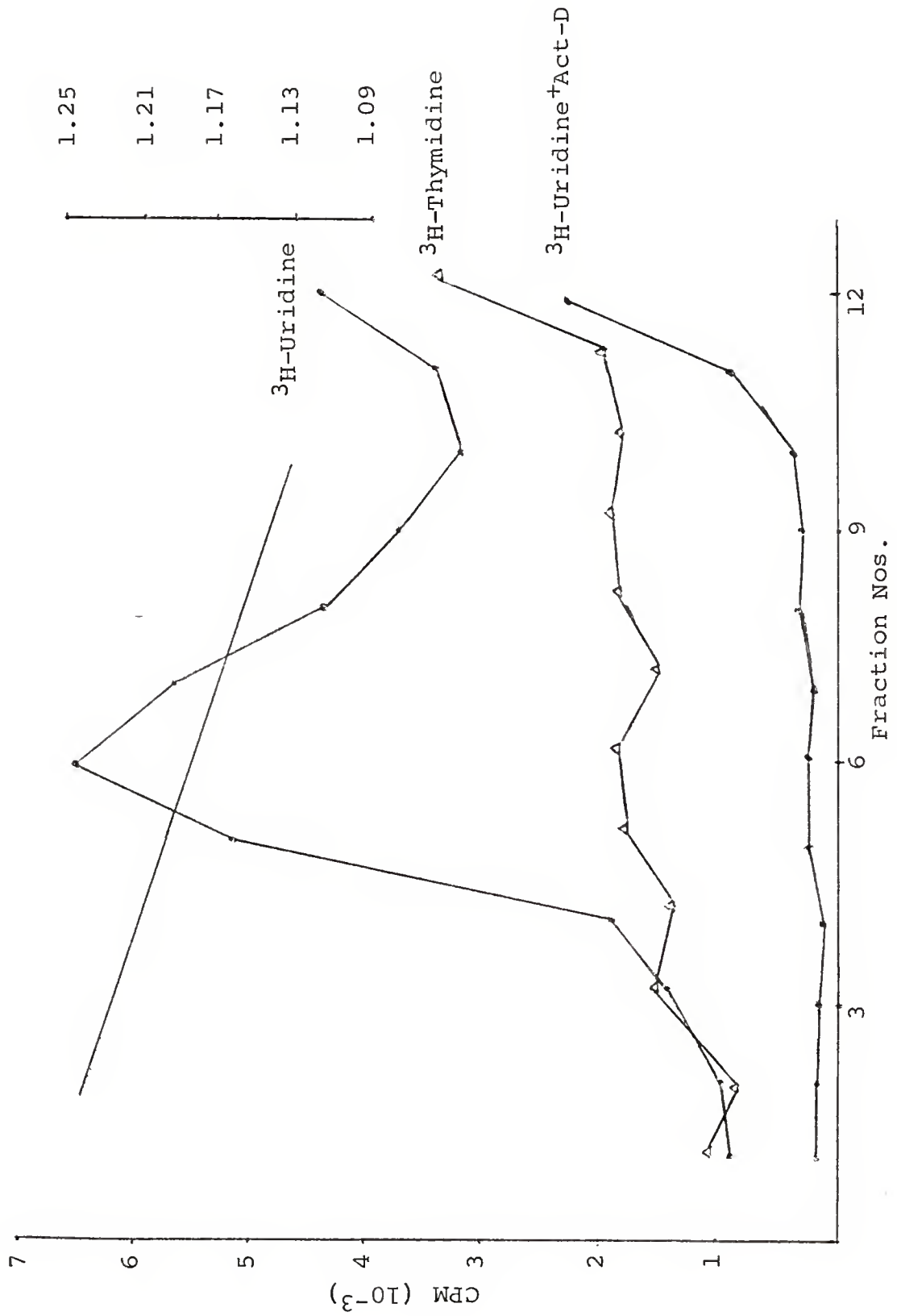
**Since the protocol for chloroform and ether treatment required 16 hours at 4^oC, the effect of such a treatment alone was determined.

2. Uridine uptake and actinomycin D: The foamy viruses incorporate uridine and not thymidine like all other RNA viruses. But the incorporation of uridine is inhibited by actinomycin D which is a characteristic of the retroviruses. This inhibition led to the belief that this group of virus go through a DNA replicative intermediate stage and to the postulation of the existence of the reverse transcriptase.

Parks et al. (26) have shown that the simian foamy agents incorporate tritiated uridine but not thymidine and band on a sucrose gradient at a buoyant density of 1.16 g/cm^3. This band did not form if the virus was grown in the presence of low levels of actinomycin D.

BHK cells infected with RSFV were incubated with ^3H-thymidine, ^3H-uridine and ^3H-uridine plus actinomycin D. Supernatant fluids containing the labeled virus were concentrated and layered on top a 15-55% sucrose gradient and spun at 200,000 gs for 2 hours. Fractions were then collected and counted. The virus incorporated the ^3H-uridine but not the ^3H-thymidine (see Fig. 18). The peak activity was at a buoyant density of 1.18 g/cm^3 similar to that reported for the feline agent. The band did not form when virus was grown in the presence of low levels of actinomycin D (2 ug/ml).

Fig. 18. Incorporation of ^{3}H-Uridine or ^{3}H-Thymidine and effect of actinomycin D on uridine incorporation. Cultures were incubated with the labeled compounds for 24 hours. Actinomycin D (2 ug/ml) was added 4 hours prior to the labeled uridine. Supernatant fluids were concentrated and layered on top of a 15 to 55% sucrose gradient and centrifuged (200,000 gs for 2 hours) using a Beckman S W 50.1 rotor. Fractions were collected and counted.


1.25
1.21
1.17
1.13
1.09
^{3}H-Uridine
^{3}H-Thymidine
^{3}H-Uridine+Act-D
CPM (10^{-3})
7
6
5
4
3
2
1
3
6
9
12
Fraction Nos.

I. Reverse Transcriptase Assays

The bovine, feline and some of the simian foamy viruses have been shown to have RNA-dependent DNA polymerase activity (17). Supernatant fluids from cultures infected with RSFV were concentrated one hundred-fold and filtered through .3 micron filters. Reverse transcriptase assays were carried out on the viral concentrate under conditions described by Parks et al. (26). The raccoon agent was shown to have virion-associated RNA-dependent DNA polymerase (see Fig. 19).

Parks et al. (26) also reported that optimal concentration of triton X-100 for demonstration of simian foamy virus type 3 reverse transcriptase activity was .1 to .2% instead of .014% as has been reported for murine leukemia viruses. Therefore the effect of varying the triton X-100 from .02 to .2% was studied, but was found to be minimal (Table VI). More characteristic of the virion-associated reverse transcriptase activity is the requirement for manganese ion which cannot be substituted for by magnesium ion (29). Table VI shows that the reverse transcriptase of RSFV had an absolute requirement for Mn^{++}.

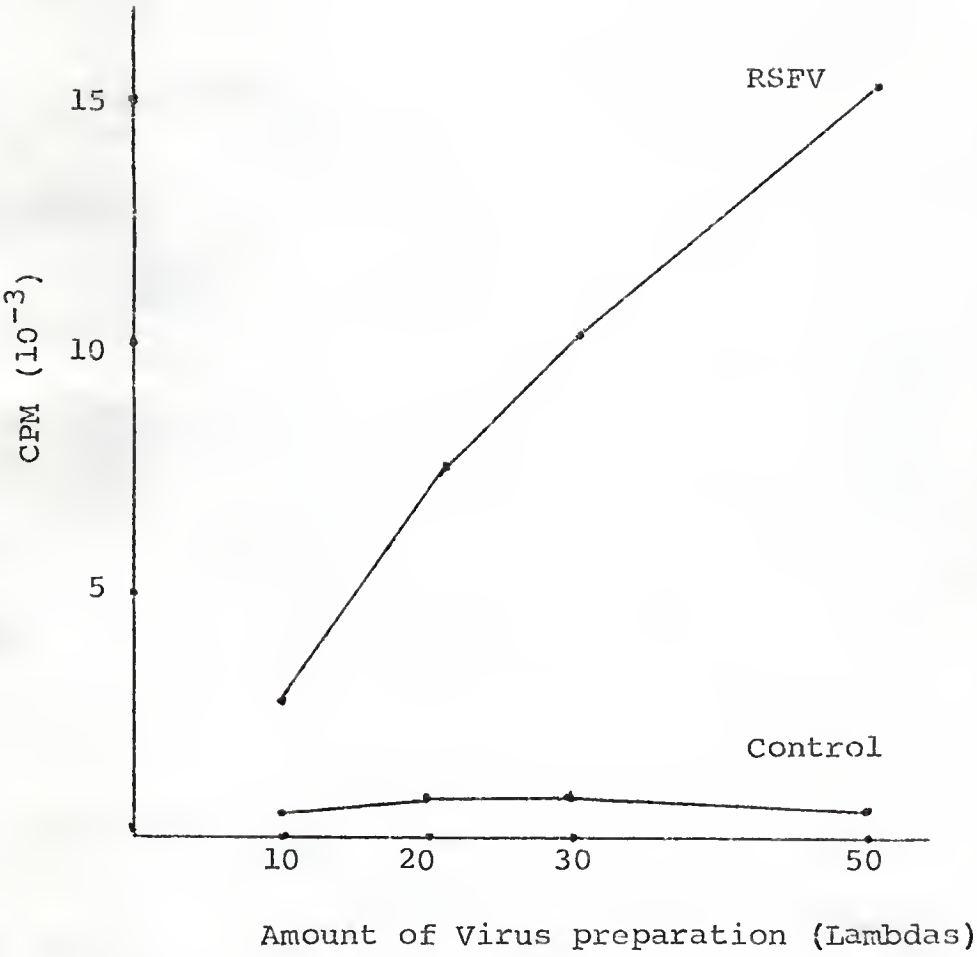


Fig. 19. Reverse Transcriptase Assay with concentrated raccoon sycyntium-forming virus--Graph shows effect of increasing virus concentration. Control shows results obtained with uninfected BHK cells treated in the same manner as the infected cells.

Table VI

Requirement for Manganese in R.T. Assay; Also Shows Effect of Varying Concentration of Triton-X 100

Substrate	Ion	T.X.100	CPM
Control	Mn++	.2%	163
RSFV	Mn++	.2%	3640
RSFV	Mn++	.02%	3188
RSFV	Mg++	.2%	244
RSFV	Mg++	.02%	249
Blank	Mn++	.2%	140

Attempts to recover reverse transcriptase activity after sucrose gradient centrifugation with simian foamy virus type III showed clearly three separate peaks of activity (26). The enzymatic action was recovered at sucrose densities of 1.22 g/cm^3 (thought to be viral cores), 1.16 g/cm^3 (the viral fraction), and of 1.08 g/cm^3 which the authors termed the soluble fraction.

The RSFV concentrate was layered on top a 15 to 60% sucrose gradient and spun at 200,000 gs for 2 hours. Fractions were collected and assayed for reverse transcriptase (Table VII). Peaks of activity were detected at 1.29 g/cm^3, at 1.18 g/cm^3 and at 1.07 g/cm^3.

J. Electron Microscopy

The foamy viruses are enveloped spherical viruses differing slightly in size. Intracellular particles are 35 to 50 nm in diameter and consist of an electron dense ring surrounding an electron-lucent center. Completed virions are seen extracellularly or within cytoplasmic vacuoles and have a diameter of 100 to 140 nm. The envelopes are covered with surface projections or spikes.

Electron microscopic studies on SIRC cells infected with RSFV revealed preformed cytoplasmic particles (different

Table VII

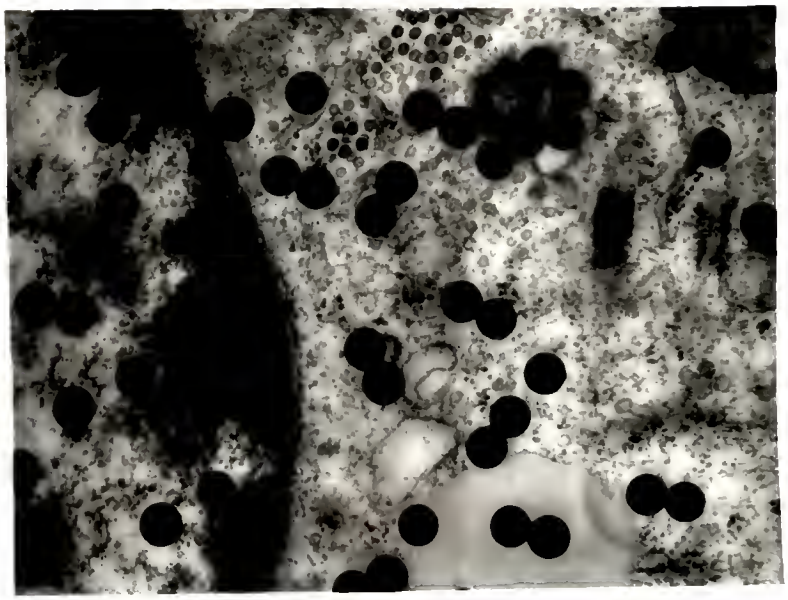
Recovery of Viral Reverse Transcriptase Activity from a Sucrose Gradient

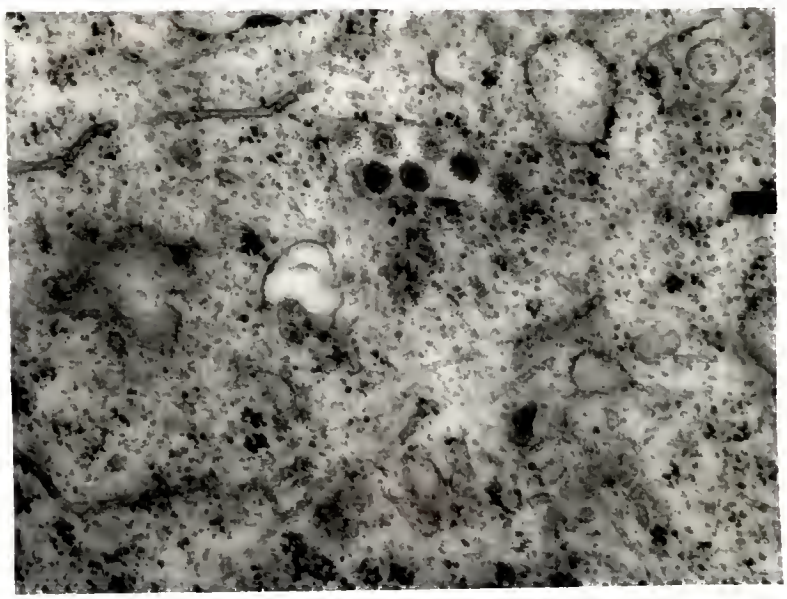
Fraction Number	Counts/min	% Sucrose	Relative Buoyant Densities
1	679	59	1.28
2	1362	57.7	1.29
3	717	54.9	1.25
4	600	52.5	1.24
5	679	50.9	1.23
6	856	49	1.22
7	901	47	1.21
8	830	45	1.20
9	650	43	1.19
10	1123	40	1.18
11	726	38	1.17
12	849	36	1.16
13	923	32	1.14
14	850	29	1.12
15	890	25.5	1.10
16	761	22	1.09
17	1234	18	1.07
Blank	850	-	-

Viral concentrate was layered on top a 15-60% sucrose gradient and spun at 200,000 gs for 2 hours using a Beckman S W 50.1 rotor. Fractions were collected and 50 lambda of each assayed for R.T. activity. The % sucrose and buoyant density of the fractions were determined on a refractometer. Peak activities are underscored.

Fig. 20. Electron microscopy showing preformed intracytoplasmic viral cores composed of an electronlucent center surrounded by electron-opaque shell. Their dismeters were estimated at 58 to 64 nm by comparison with beads that were 188 nm in diamter (Magnification X40,000).

Fig. 21. E. M. showing complete virions in intracytoplasmic vacuole. Diameters estimated at 85 to 105 nm. Surface projections or spikes can be seen on the outer envelopes (Magnification X50,000).





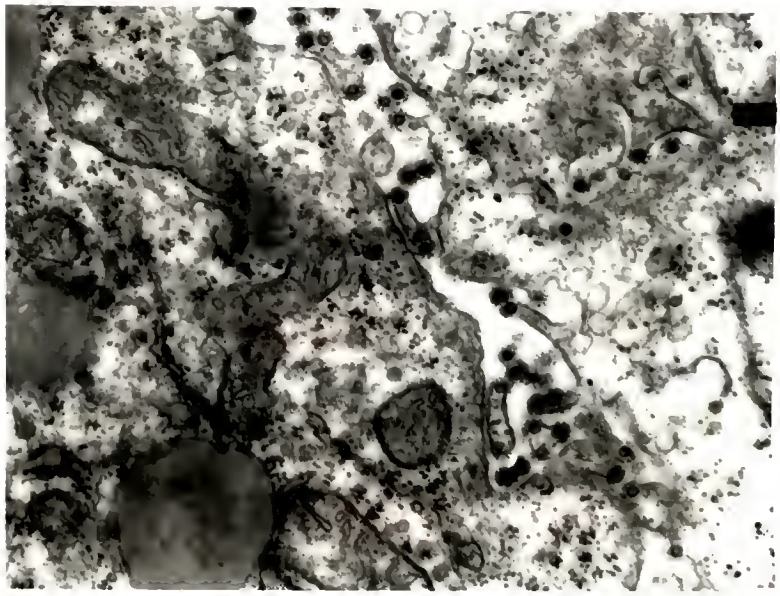


Fig. 22. E. M. showing extracellular virions. Nucleoids can be seen budding out of cell or into intracytoplasmic vacuole and acquiring their envelope in the process (Magnification X35,000).

from C type) of 58 to 64 nm in diameter (see Fig. 20), as estimated by comparison with beads that were 188 nm in diameter. Completed enveloped RSFV could be seen inside cytoplasmic vacuole (see Fig. 21). Extra cellular virions and virions observed in cytoplasmic vacuoles were 85-105 nm in diameter (see Fig. 22). Preformed viral cores could be seen to mature acquiring their outer envelope by budding out of the cell or into cytoplasmic vacuoles (see Fig. 22). The envelope was observed to be covered with small projections.

The RSFV has all of the morphological characters ascribed to the members of the Spumavirinae. The morphogenesis of RSFV as observed by E.M. was more like that of the myxoviruses than of the C-type oncornaviruses.

K. Serological Survey

The foamy viruses are ubiquitous in nature causing persistent infection in their respective hosts. As an example, Hackett et al. (16) reported isolation of feline syncytium-forming virus from 90% of the cats studied in their laboratory during the first four months of 1969. Since the foamy viruses are usually widespread in their hosts, a serological survey of Florida raccoons was made

to study the frequency of occurrence and geographical distribution of RSFV. Sera from numerous counties were provided by Dr. William Bigler of the Florida Department of Health and Rehabilitative Services in Tallahassee, Florida. The survey (Table VIII) indicated that infection with RSFV is widespread in the raccoon population of Florida.

Table VIII

Survey of Raccoon Sera for Precipitating Antibodies to RSFV; Geographical Distribution by Counties

Counties	Number Tested	Number Positives
Alachua	8	3
Clay	2	1
Collier	68	39
Columbia	4	0
Dade	4	1
Duval	5	1
Levy	3	0
Monroe	3	0
Polk	2	2
Sarasota	16	9
Suwannee	5	3
Total	120	56

V. DISCUSSION

The raccoon syncytium-forming virus described herein has all of the distinctive characteristics of the other members of the Spumavirinae. The cythopathology produced in infected cells was manifested principally in formation of syncytia. Intense nuclear staining by indirect immunofluorescence showed production of viral antigen in the nuclei of these cells, a main difference with the other members of the Retroviridae and the human and bovine respiratory syncytial viruses. Electron microscopy revealed a morphology and morphogenesis distinct from the C-type particles.

RSFV nucleocapsids are first observed in the cytoplasm and migrate to the cell membrane or intracytoplasmic membranes, acquiring an envelope by budding. In contrast, the nucleocapsids of the C-type oncornaviruses form at the plasma membrane as the viruses bud through. Electron microscopic studies of the feline and bovine agents have indicated that nucleocapsids are assembled in the nuclei of infected cells (17). Immunofluorescent studies of all of the foamy agents

thus far examined demonstrate that viral antigens are initially detected in the cell nuclei (17). In contrast, cells infected with C-type particles show membrane and cytoplasmic fluorescent staining.

A precipitating antigen preparation extracted from infected cells was used to screen raccoons for infection with RSFV. This antigen may be a viral structural protein or a protein produced in the course of viral infection that is not incorporated into the virus. Since viral antigens are predominantly observed in the nuclei of infected cells by immunofluorescence and because electron microscopic studies indicate that nucleocapsids are formed in the nucleus or perinuclear cytoplasm, it is likely that it is this antigen which is responsible for the precipitating activity since immunodiffusion is less sensitive than immunofluorescence. Attempts to demonstrate that the antigen is a viral structural protein did not succeed. Ultracentrifuged concentrates of cell-free virus did not react by immunodiffusion. It is possible, because RSFV replicates to low titers, that the ultracentrifuged virus preparations were not sufficiently concentrated to give a visible precipitin band. Another possibility, that the antigen is a non-structural virus protein, could also be true. A third hypothesis, that the

antigen is a structural protein subunit of which the reactive site is hidden when the nucleocapsid is fully assembled, is also possible. The nature of this antigen preparation is not addressed in the literature on the foamy viruses and needs more study.

As can be observed in Figures 7 and 8, either one or two precipitation bands were produced by immunodiffusion with the antigen extract. Raccoon and rabbit antisera gave similar reactions and, when sera from each species were placed in adjacent wells, the lines which formed upon reaction with the antigen were observed to fuse, indicating identity. When two lines formed, one was always more pronounced than the other. The major line was sometimes observed to be nearer the antigen well, sometimes nearer the antiserum well. The relative location or distribution of the two lines appeared to vary with the antigen preparation used.

The raccoon agent incorporated tritiated uridine but not thymidine and this incorporation was inhibited by actinomycin D. This indicated that RSFV is an RNA virus requiring a DNA replicative intermediate. The high background of tritiated thymidine recovered throughout the gradient (see Fig. 18) probably reflects cellular DNA

synthesis. Raccoon syncytium-forming virus was shown to possess virion-associated RNA dependent DNA polymerase. Only weak bands of activity were recovered from fractions collected after sucrose density gradient centrifugation. This activity, however, was found at the same densities in several repetitions of the experiment, and was distributed in the same fashion as the reverse transcriptase activity of the simian foamy viruses as described by Parks et al. (26).

Work with the foamy viruses of various species has shown that they are common in nature, cause persistent infections, and persist in the leukocytic cells of their respective animal hosts. Scott (30) concluded that FeSFV was a nuisance to virologists, oncologists, and vaccine producers working with feline cells. Hackett and Manning (15) point out that since this agent is strongly cell-associated and replicate to such low titers it can easily be overlooked.

Much significance has been placed on the discovery of reverse transcriptase in cell of humans and other animals. Until recently reverse transcriptase was considered to be an exclusive property of the tumor producing oncorna viruses. The foamy viruses share the following properties with that group (25):

1. Similar buoyant densities.
2. Virion-associated reverse transcriptase.
3. Inhibition of replication by BUDR and dactinomycin.
4. High resistance to inactivation by ultraviolet irradiation.

They differ with respect to:

1. Formation of syncytia as characteristic CPE.
2. Production of an intranuclear antigen detectable by immunofluorescence.
3. Unique morphogenesis as determined by electron microscopy.
4. No cellular transformation in vitro or tumor production yet observed.

As Parks and Todaro (25) so aptly point out this last difference may not be absolute. All cells thus far tested have supported viral replication. A non-permissive system has not been studied. Such systems may be subject to transformation by the foamy viruses. Their ubiquitous nature and persistence in their hosts makes it important to find ways to readily detect these viruses. As pointed out earlier, one such virus (simian foamy virus Type I) has been shown to induce a carrier state in certain cells. These viruses can contaminate cell cultures and be responsible for enzyme activities associated with oncogenesis.

Since the syncytium-forming viruses of different species have been shown to have similar biological properties, such studies may very well help in understanding the newly isolated human agent. Lately some researchers have been studying reverse transcriptase in normal human lymphocytes. From experience with other species, a syncytium-forming virus could be responsible for this activity. There should be a concerted effort to uncover and characterize these viruses so that virologists, cancer researchers, and vaccine producers can be made more aware of them.

APPENDICES

APPENDIX I

FITC--CONJUGATION OF SERUM

FITC--CONJUGATION OF SERUM

(As cited in Gaskin (1973)(11))

1. To a two-fold dilution of serum in phosphate buffered saline (.01 M phosphate, .15 M NaCl, pH 7.2), add an equal volume of saturated ammonium sulfate, stirring constantly with a magnetic stirrer. Continue stirring for 1 hour.
2. Centrifuge 1400 g for 20 minutes.
3. Dissolve pellet in PBS to the original volume.
4. Add dropwise with continuous stirring 1/2 volume of saturated ammonium sulfate. Continue stirring for 1 hour.
5. Centrifuge 1400 g for 20 minutes.
6. Dissolve pellet in distilled water to 1/2 the original serum volume.
7. Dialyse, with stirring, against PBS at $4^{o}C$ using at least 5 changes of at least 50 times volume per change. Clarify dialysate by centrifugation.
8. Read absorbance of a 1:100 dilution of dialysate at a wavelength of 276 nm in a spectrophotometer.

   Reading X 100 ÷ 1.37 = protein concentration in mg/ml.

10. Make .5 M carbonate buffer, pH 9.5 as follows: 3.7 gm $NaHCO_3$, 0.6 gm anhydrous Na_2CO_3, made up to 100 ml with distilled water.

    To a volume of globulin solution (20 mg/ml), add 1/2 volume of carbonate buffer containing 0.013 mg FITC for each mg of protein to be conjugated.

11. Stir at 4^oC for 6-10 hours.

12. Pass immediately through medium or coarse Sephadex G-25 equilibrated with PBS; one cm of a 2.5 X 40 cm column for each ml of conjugate. Elute with PBS and collect the first yellow band.

13. Dialyse the conjugate against .1 M Tris-HCl buffer, pH 7.8 until equilibrated (4^oC). Pass through DEAE-Sephadex (2 cm of a 2.5 X 40 cm column per ml of conjugate) equilibrated with the same buffer.

    Elute with .1 M Tris-HCl, pH 7.8

    .1 M Tris-HCl + .1 M NaCl, pH 7.8

    .1 M Tris-HCl + .2 M NaCl, pH 7.8

    .1 M Tris-HCl + .3 M NaCl, pH 7.8

    Collect each fraction separately, monitoring the collection with a black light as necessary.

14. Concentrate the fractions by dialysis against polyvinylpyrrolidone to a volume equivalent to the original serum volume or less.

15. Test the fractions, if possible, by setting up an immunodiffusion test against the antigen concerned. Formed precipitin lines should fluoresce under black light after the test has been washed overnight in PBS to elute most unreacting conjugated proteins.

    Determine the molecular fluorescein:protein ratio by reading a 1:50 or other suitable dilution in a spectrophotometer at 280 and 495 nm, and employing the nomograph of The and Feltcamp.* A molecular F:P ratio of 1-4 is supposed to be optimal, giving good specific fluorescence while minimizing non-specific fluorescence.*

16. Add 1:10,000 merthiolate if desired. Centrifuge the fractions at high speed (e.g., 10,000 g) for clarification. Store at -20° to -70°C in small aliquots.

    Test dilutions of conjugate on acetone-fixed infected cover slip preparations to determine the efficacy of the conjugate and the optimally effective dilution for use.

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*The, T. H. and T. E. W. Feltcamp. Conjugation of fluorescein isothiocyanate to antibodies. II. A reproducible method. Immunol. 18:875-881 (1970).

APPENDIX II

ERIOCHROME BLACK: A COUNTERSTAIN FOR IMMUNOFLUORESCENCE

ERIOCHROME BLACK A COUNTERSTAIN FOR
IMMUNOFLUORESCENCE
(As cited by Gaskin, 1973 (11))

I. Chelating Agent

A.	1.	N, N-dimethylformamide	200 ml
	2.	Distilled H_2O	80 ml
	3.	0.1 M Aluminum Chloride (2.4 gm/100 ml)	40 ml
	4.	1 M Acetic Acid (1 M = 5.7 glacial acetic acid/100 ml H_2O)	40 ml
		Total	360 ml

B. Adjust to pH 5.2 with 1 M sodium hydroxide (1 M = 4 gm NaOH/100 ml H_2O - need approx. 10 ml (?)

C. Make up to total of 400 ml with distilled H_2O

II. Preparation of Dye:

1. Weigh .628 gm Eriochrome Black A

2. Add dye to 80 ml N, N-dimethylformamide

3. Add 400 ml chelating agent slowly while swirling the dye solution

III. Dilute 1:5 or more with distilled water as necessary

Stain tissue culture 1-3 seconds

Stain tissue sections 10-30 seconds

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BIOGRAPHICAL SKETCH

Stanley Nicolas Laham was born in Jacksonville, Florida, on June 17, 1950. He completed his primary education at the Institution Saint Louis de Gonzague in Port-au-Prince, Haiti, and his secondary education at the P. K. Yonge Laboratory School in Gainesville, Florida. He entered Jacksonville University in 1966 and transferred to the University of Florida in 1967 where he specialized in international relations. He obtained his Bachelor of Arts degree in political science in 1970. He completed his studies in the Department of Immunology and Medical Microbiology of the College of Medicine and received the degree of Doctor of Philosophy.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Jack M. Gaskin, Chairman
Assistant Professor of Preventive Medicine and Immunology and Medical Microbiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Kenneth I. Berns
Professor and Chairman of Immunology and Medical Microbiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

William Hauswirth
Assistant Professor of Immunology and Medical Microbiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Richard B. Grandall
Professor of Immunology and Medical Microbiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Philip Laipis

Philip Laipis
Assistant Professor of Biochemistry
and Molecular Biology

This dissertation was submitted to the Graduate Faculty of the College of Medicine and the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

June 1978

Dean, College of Medicine

Mildred A. Hall

Dean, Graduate School